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CHANGES IN MITOCHONDRIAL ACTIVITY CAUSED BY AMMONIUM SALTS AND THE PROTECTIVE EFFECT OF CARNITINE

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SUMMARY: Ammonium salts added to isolated rat liver mitochondria deviate α-ketoglutarate to glutamate synthesis, thus decreasing its availability as respiratory substrate. As a consequence a decrease of respiratory rate is observed which is paralleled by progressive mitochondrial swelling. It was demonstrated that L-carnitine may abolish this swelling thus improving structural and metabolic state of mitochondria.

A number of studies have conclusively shown that L-carnitine plays an important role in lipid metabolism being essential for the optimal oxidation of fatty acids by forming a shuttle system with Acyl-CoA (for review see 1). This fact may explain the observation that L-carnitine ameliorates the functional properties of mitochondria in a number of situations associated with high concentrations of fatty acids (2 - 6). In fact, high concentrations of acyl-CoA, especially long-chain acyl-CoA, are known to induce alterations in the mitochondrial membranes (7,8). Moreover, L-carnitine may improve the mitochondrial function also by replenishment of CoA into intramitochondrial space during acyl-carnitine transport out of mitochondria (9 - 11).

Less immediately apparent is the mechanism by which L-carnitine partially prevents the inhibition of mitochondrial respiration caused by *in vivo* treatment with ammonium acetate (12). The problem is even more complicate by a fact that the precise mechanism of toxicity of ammonia is still debated

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(13,14,15). Our previous findings have shown that ammonium acetate treatment *in vivo* adversely affects the functioning of liver mitochondria (12); however the site of this effect is still unknown.

In order to get better insight into the specifity of this intoxication and its prevention by carnitine, isolated rat liver mitochondria were treated *in vitro* with ammonium acetate and related compound, and the effect of carnitine was investigated in these experimental models.

MATERIALS AND METHODS

Male albino rats of Sprague-Dawley strain weighing 200-230 g maintained on the standard diet ad water *ad libitum* were used. Liver mitochondria were prepared and their respiratory parameters were measured as indicated in (16). Respiratory states were those defined by Chance and Williams (17).
Glutamate was measured in neutralized perchloric acid extract using enzymatic method (18). Modification in the average dimensions of isolated mitochondria were estimated by determining the variations of the dissymetry light scattering at different angles (19). The dissymetry was defined as the ratio of the intensity of light scattered at 45° toward that of light scattered at 135°, by using a Model 5000 Light Scattering Monophotometer (C.N.Wood M.F.G. Co, Newton, Pa). The wave length was 546 nm.
The concentration of proteins was determined by the Biuret method (20).
All reagents were of pure analytical grade. Enzymes were purchased from Boehringer-Manheim. Amrinone (5-amino-3,4'-bipirydin-6(1H)-one; Win 40680) was from Winthrop-Breon (USA). L-carnitine was of Sigma-Tau (Pomezia, Italy).

RESULTS

Fig.1 shows that *in vitro* addition of ammonium acetate to isolated liver mitochondria incubated under the conditions of state 4 respiration, causes a rapid loss of respiratory control. The control is almost completely abolished when the concentration of ammonium acetate reaches 2 mM. The same figure shows that a similar effect is induced by addition of ammonium chloride. On the contrary, addition of sodium acetate at the same concentrations is without any appreciable effect. The respiratory inhibition of state 3 is associated with a decrease in the phosphorylative efficiency as indicated by the progressive decrease of ADP/O

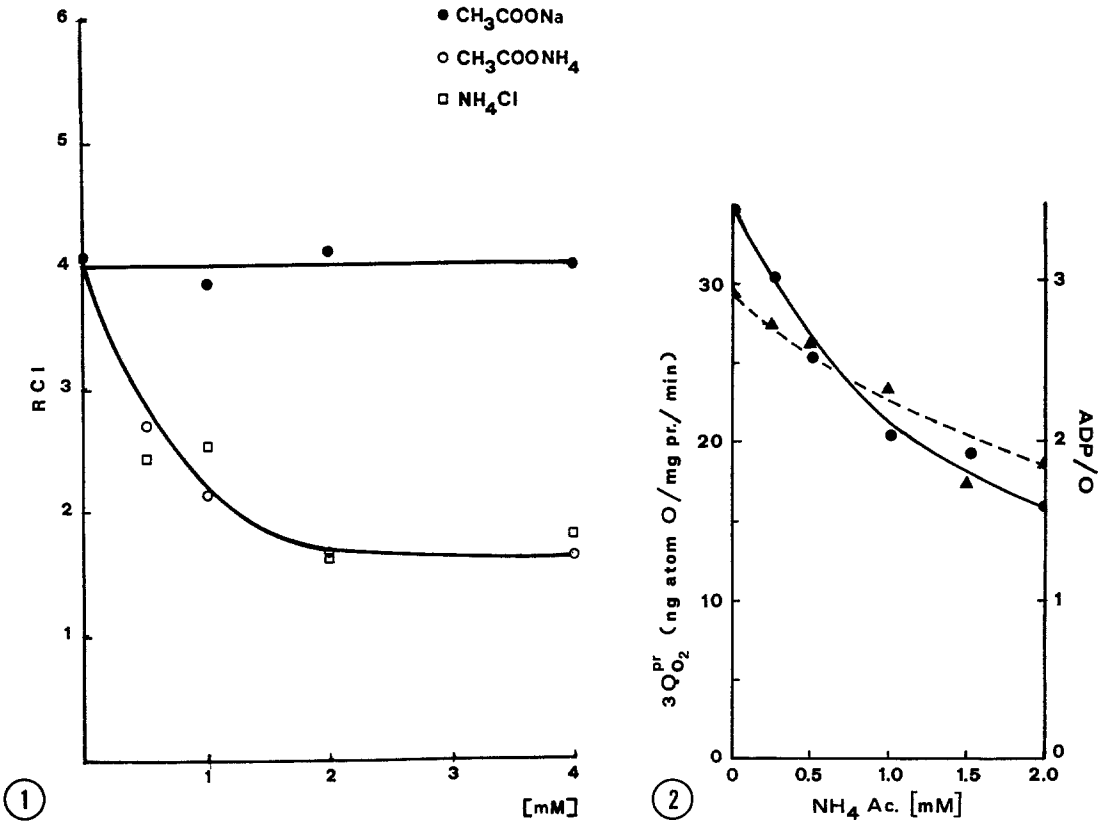

Fig.1 Effect of sodium acetate, ammonium acetate and ammonium chloride on respiratory control index of isolated rat liver mitochondria.

Mitochondria were isolated and their respiratory parameters were measured as indicated by Bobyleva-Guarriero et. al (16) at 30° C.

Fig.2 Effect of ammonium acetate on isolated rat liver mitochondria.

Mitochondria were isolated from 24h fasted rats. Respiratory substrate was 2mM citrate. Results of one representative experiment.

●——● - rate of respiration in state 3
▲- - -▲ - ADP/O ratio

ratios (Fig.2). The highest inhibition of oxygen uptake was obtained with citrate and α-ketoglutarate, whereas the same mitochondria incubated in the presence of succinate did not undergo any significant change after ammonium acetate addition (Table 1). The data of Table 1 indicate that some block at the level of α-ketoglutarate may exist. In fact, α-ketoglutarate may be deviated by excess of ammonia to glutamate formation thus decreasing the

Table I. Effect of ammonium acetate on the oxygen uptake in state 3 in mitochondria respiring with different substrates

	ng atoms oxygen /mg proteins /min			
Treatment	Pyruvate	Citrate	α-ketoglutarate	Succinate
CONTROL	49.2 ± 6.2	46.3 ± 6.4	76.2 ± 25.0	122 ± 42.0
+Ammonium acetate	41.1 ± 8.5**	23.3 ± 3.5*	47.4 ± 6.7**	128 ± 47.0

Mitochondria were prepared from 24 hours fasted rats liver.
Substrates were 2 mM. 1 mM ammonium acetate was added into state 4.
Values represent media ±SD of 3-4 experiments.
* $p < 0.001$ with respect to control.
** $p < 0.05$ with respect to control.

flow through tricarboxylic acids cycle. Table 2 shows that the addition of ammonium acetate to mitochondria incubated in the presence of citrate and ADP significantly increases the glutamate production with respect to control. The addition of amrinone, a powerful inhibitor of glutamate dehydrogenase (21) to incubation mixture completely inhibits glutamate production by mitochondria in the presence of ammonium acetate (Table 2) and simultaneously induces the partial release of inhibition of oxygen uptake. The same Table also shows that whereas the addition of carnitine does not modify glutamate concentration in the presence of ammonium acetate, the inhibition of oxygen uptake due to ammonium acetate is partially removed in its presence. The rate of oxygen consumption in state 3 of mitochondria incubated in the presence of L-carnitine, amrinone and ammonium acetate is comparable with that of control mitochondria (Table 2). Fig. 3 illustrates the effect of ammonium acetate on structural steady-state of mitochondria in absence and in the presence of L-carnitine. The variation of mitochondrial structure was estimated by measuring the variation of dissymetry of light scattered at different angles that increases linearly with the average volume of parti-

Table II. Glutamate production and oxygen uptake by rat liver mitochondria in vitro

Treatment	Glutamate nmole/mg prot.	Rate of Oxygen uptake in state 3 ngA O/mg prot./min (0 — 20 — 40 — 60)
Control	2.83 ± 0.81	100%
+Amm.acetate	5.12 ± 1.61*	55%
+ Amrinone	0	121%
+Amm.acetate +amrinone	0	81%
+L-carnitine	2.79 ± 1.00	108%
+L-carnitine +Amm.acetate	5.29 ± 1.14*	76%
+L-carnitine +amrinone +Amm.acetate	-	98%
Time 0	2.03 ± 1.00	

Glutamate determination: Total volume of incubation mixture was 1.20 ml. Citrate was 2 mM, Ammonium acetate - 1 mM, L-carnitine - 1 mM, mitochondria: 30-40 mg/ml; ADP - 5 μmoles/ml; amrinone was 0.08 mg/ml. Temperature of incubation was 30°C. The reaction started by addition of mitochondria. The incubation was stopped after 2 minutes adding 0.12 ml of 60% $HClO_4$, the tubes were centrifuged at 5000xg for 15 min and the supernatantes were neutralized with 20% KOH containing 0.3 M MOPS pH 7.4. Glutamate was measured by method indicated in Materials and Methods.
Values represent media ±SD of 7 experiments.
* $p < 0.001$ in comparison with control
Oxygen uptake was measured as indicated in (16) in the presence of 2mM citrate, ammonium acetate and L-carnitine were 1mM, amrinone was 30 μg/ml, mitochondria were 3.3 mg prot/ml. The data of one representative experiment are given.

cles. The figure shows that addition of ammonium acetate to mitochondria respiring under state 1, induces large increase in mitochondrial swelling. If L-carnitine is present in the incubation medium, further addition of ammonium acetate fails in inducing mitochondrial swelling; the mitochondria appear to maintain the volume seen in the control.

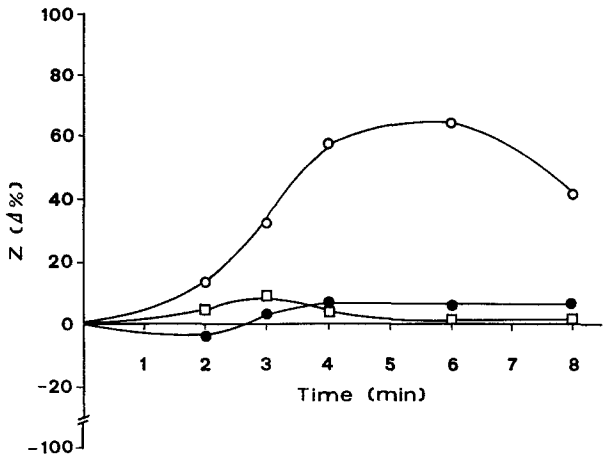

Fig. 3 Effects of ammonium acetate and L-carnitine on the structural steady-state of isolated rat liver mitochondria.

Rat liver mitochondria were prepared from 24 h fasted animals. The variations of mitochondrial dimensions are revealed by the dissymetry of the light scattered by mitochondria at different angles. Increase of dissymetry, Z, indicates swelling of mitochondria.
The dissymetry is measured by ratio of intensity of light scattered at 45° to that of light scattered at 135°.
Mitochondria (5 µg of protein/1ml) were incubated under the conditions of state 1 at room temperature.
the wave length was 546 nm.

●——● Control
○——○ +5mM Ammonium acetate
□——□ +5mM L-carnitine+5mM Ammonium acetate.

DISCUSSION

The results of the present research confirm those obtained previously *in vivo* (12), i.e. ammonium salt interferes with a mechanism of energy transformation at the mitochondrial level depressing both respiratory activity and phosphorylation efficiency.

This effect is due to ammonium because either ammonium chloride or ammonium acetate have the same inhibitory activity, whereas sodium acetate has no effect (Fig.1). Since both *in vivo* (12) and *in vitro* observations show that oxygen uptake is impaired with α-ketoglutarate and with respiratory substrates preceding it in tricarboxylic cycle as well (Table 1), but not with succinate, it may be supposed that a part of α-ketoglutarate is consumed in

the reaction of glutamate formation due to excess of ammonium. Indeed, the amount of glutamate formed by mitochondria in the presence of ammonium acetate is increased. The above indicated deviation of α-ketoglutarate impaires the activity of tricarboxylic acids cycle. Moreover, glutamate formation requires also NADH thus decreasing its supply to respiratory chain. The partial release of respiratory rate by amrinone (Table 2), which completely inhibits the glutamate formation, confirms this hypothesis.

As a consequence of decrease in respiration, the mitochondria undergo swelling, most probably due to acetate and /or phosphate penetration into mitochondria (22-24) (Fig 3.). L-carnitine counteracts this swelling thus increasing the respiratory activity of mitochondria (Table 2).

The results of the present research confirm that the effect of carnitine occurs at the level of mitochondria, but they also indicate that the mechanism of this protection against the damages by ammonium acetate is more complex than that proposed by O'Connor and coworkers (25). According to these authors carnitine enhances the transport of Acyl-CoA into mitochondria, thus increasing the intramitochondrial reducing equivalents and improving the function of malate-aspartate shuttle depressed by hyperammoniaemia (23). The consequence would be an increased disposal of energy compounds capable of reversing the effect of ammonium acetate. This may be true in vivo, i.e., in a condition in which supply of Acyl-CoA from other comparts to mitochondria does occur. However, the present data show that carnitine may counteract the effect of ammonium also when added in vitro, i.e. under conditions in which mitochondria hardly utilize fatty acids. In fact, under these conditions, the mitochondria use either endogenous substrates or the substrates added to the flask.

Whichever the mechanism might be, the present data indicate that carnitine may prevent mitochondrial swelling, thus ameliorating both the structural and the functional integrity of mitochondria. The mechanism of this effect is under study.

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